JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

HIV Protease-Mediated Activation of Sterically Capped Proteasome Inhibitors and Substrates

Dennis L. Buckley,[†] Timothy W. Corson,[‡,∥] Nicholas Aberle,[‡] and Craig M. Crews*[,†,‡,§]

[†]Department of Chemistry, [‡]Department of Molecular, Cellular & Developmental Biology, and [§]Department of Pharmacology, Yale University, New Haven, Connecticut 06511, United States

Supporting Information

ABSTRACT: Strategies for selectively killing HIV-infected cells present an appealing alternative to traditional antiretroviral drugs. We show here the first example of an inactive "Trojan horse" molecule that releases a cytotoxic, small-molecule proteasome inhibitor upon cleavage by HIV-1 protease. As a proof-of-concept strategy, the protein avidin was used to block entry of the compound into the proteasome in the absence of HIV-1 protease. We demonstrate that this strategy is also feasible without requiring an exogenous protein; a polylysine dendrimer-containing molecule is unable to enter the proteasome until cleaved by HIV-1 protease. These results demonstrate that conditional proteasome inhibitors could prove useful in the development of new tools for chemical biology and future therapeutics.

The development of anti-HIV drugs, including reverse transcriptase inhibitors, protease inhibitors, co-receptor inhibitors, and integrase inhibitors, has changed HIV infection into a chronic, mostly treatable condition.[1] However, drug resistance is problematic, and this is exacerbated by the need for continual antiretroviral treatment.[2,3] As an alternative to the simple management of long-term HIV infection, strategies have been developed that seek to exploit cleavage by HIV-1 protease to activate "Trojan horses" that selectively kill HIV-infected cells. Previously, these strategies have employed proteins such as caspases,[4] proteinaceous toxins,[5] and ribonucleases[6] that normally exist as inactive zymogens or contain a destabilizing sequence. These proteins were modified to contain an HIV-1 protease recognition sequence; cleavage by HIV-1 protease activated or stabilized the cytotoxic protein, leading to cell death.

To make this approach more drug-like, we sought to develop a compound that would release a cytotoxic small molecule instead of a protein. Our strategy involves the HIV-protease-mediated release of a cytotoxic epoxyketone proteasome inhibitor[7] that is rendered inactive by incorporating a large "blocking" group (Figure 1). This steric cap prevents entry of the inhibitor into the hollow catalytic chamber of the proteasome, which has an opening of 1.3 nm.[8,9] On the basis of a comparison of SQNY/PIV, an HIV protease consensus sequence, with structure-activity data from proteasome inhibitor design studies,[10,11] the peptide SQNY/PIVF was chosen to form a protease-labile linker between a leucine epoxyketone and the steric cap. Upon cleavage by HIV protease,

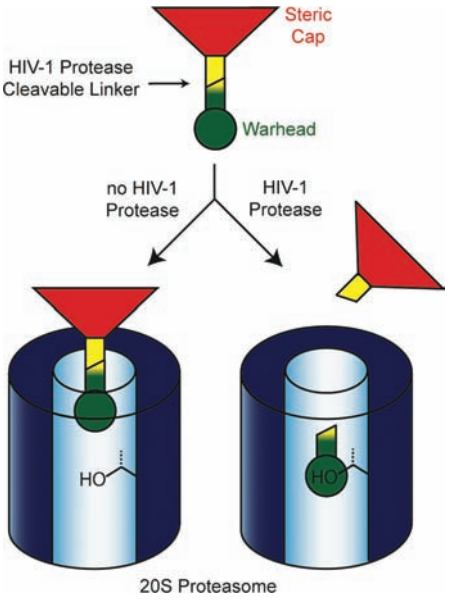

Figure 1. A "Trojan horse" that releases a small molecule capable of reaching the catalytic threonine of the proteasome only after cleavage by HIV-1 protease.

1 would be released (Figure 2a), which we have found to be a potent inhibitor of the proteasome's chymotrypsin-like activity with low nanomolar cytotoxicity, similar to other epoxyketone-based proteasome inhibitors.[11]

As a proof of concept, we synthesized **2**, incorporating a biotin moiety that, in the presence of avidin, would create a steric cap large enough to prevent entry into the proteasome (Figure 2a). Instead of including an epoxyketone pharmacophore, we first incorporated a fluorogenic aminomethylcoumarin (AMC) moiety[12] that gives a direct readout of the ability of **2** to enter the proteasome. As shown in Figure 2b, when incubated with 20S proteasomes in the absence of avidin, **2** is able to enter the proteasome, where AMC is liberated and fluoresces. However, in the presence of avidin, **2** cannot enter the proteasome, preventing AMC release. Upon addition of HIV-1 protease, an increase in fluorescence is detected, indicating that the protease-labile linker is cleaved, allowing the released fragment to enter the proteasome and AMC to be hydrolyzed by the proteasomal catalytic sites (Figure 2b).

Encouraged by this result, we next synthesized **3**, which contained a Leu-epoxyketone[13] in place of the Leu-AMC, and

Received: October 18, 2010
Published: December 27, 2010

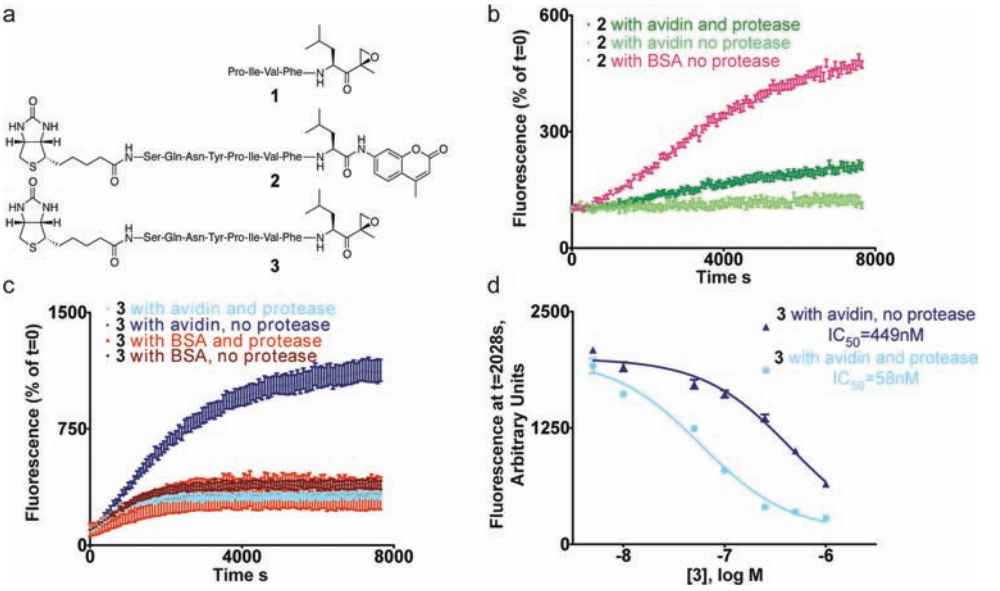

Figure 2. (a) Structures of biotin-containing compounds and the released epoxyketone proteasome inhibitor. (b) Activity of **2** as a proteasome substrate with or without pretreatment with HIV-1 protease and in the presence of avidin (0.72 mg/mL, 10 U/mL) or bovine serum albumin (BSA). Activity of proteasome substrates is determined by fluorescence of released AMC, normalized against starting fluorescence and monitored over time. (c) Activity of **3** as a proteasome inhibitor with or without pretreatment with HIV-1 protease and in the presence of avidin (0.72 mg/mL, 10 U/mL) or BSA. (d) Dose-dependent response of **3** with avidin (0.72 mg/mL, 10 U/mL), with or without pretreatment with HIV-1 protease. Activity of proteasome inhibitors is measured by inhibition of fluorescence of AMC released from Suc-LLVY-AMC, normalized against starting fluorescence and monitored over time.

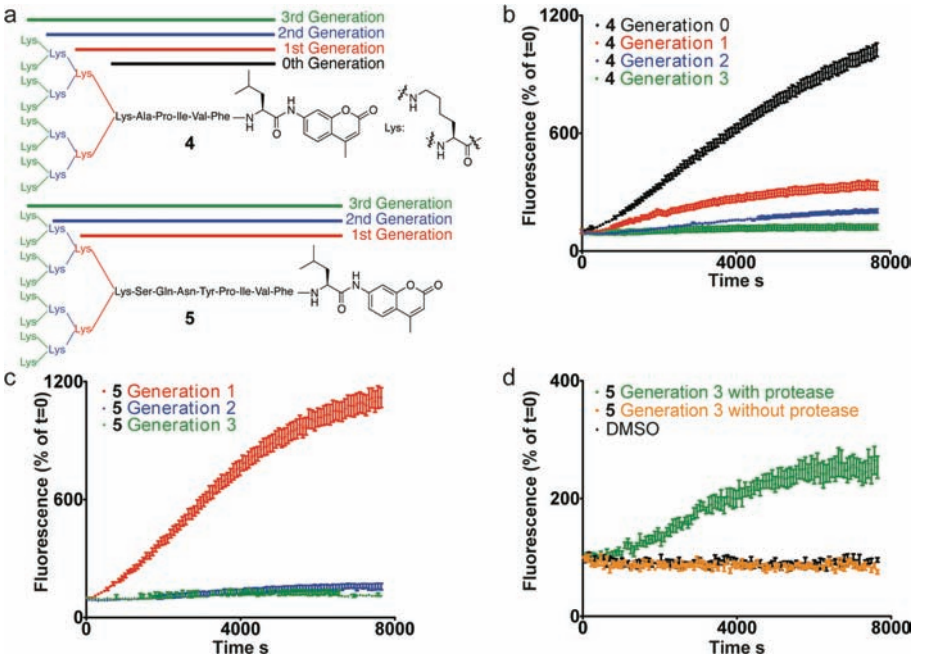

Figure 3. (a) Structures of generations 0−3 of **4** and generations 1−3 of **5**. (b) Activity of generations 0−3 of **4** as proteasome substrates. (c) Activity of generations 1−3 of **5** as proteasome substrates. (d) Activity of generation 3 of **5** with or without pretreatment with HIV-1 protease. Activity of proteasome substrates is determined by fluorescence of released AMC, normalized against starting fluorescence and monitored over time.

demonstrated that it inhibits proteasome activity (as measured by the cleavage of the fluorogenic substrate LLVY-AMC) in the absence of avidin. The addition of avidin decreased the inhibitory activity of **3**, while the addition of HIV-1 protease liberated **1**, restoring potent proteasomal inhibition (Figure 2c). The dose dependency of this effect was studied by measuring proteasome activity in the presence of various concentrations of **3** with or without exposure to HIV protease (Figure 2d). The chymotrypsin-like activity of the proteasome was inhibited by **3** with an IC_{50} of 58 nM in the presence of avidin after incubation with HIV-1 protease, a 7.7-fold increase in potency compared to the control lacking HIV-1 protease (IC_{50} = 450 nM).

dx.doi.org/10.1021/ja109377p |*J. Am. Chem. Soc.* 2011, 133, 698–700

At this point, we began to explore options that did not require an ectopic protein, which led us to dendrimers as possible steric caps. In particular, we decided to base our steric cap on dendritic polylysines, which were developed as cell-permeable transfection reagents.[14−17] While previous work by DeMartino and co-workers[18] has shown conclusively that the proteasome can accommodate more than one peptide chain, we felt it would be possible to create a large enough branched dendrimer to prevent entry into the proteasome. We synthesized generations 0 through 3 of dendritic polylysine compound **4** in order to determine the minimal size of the dendrimer needed to prevent proteasome entry (Figure 3a). We first used a shortened peptide linker, allowing us to ignore the possibility that an overly long linker might enable the AMC substrate to reach the active site inside the proteasome even if the dendrimeric end of the molecule cannot enter. Our assays showed that the ability of these compounds to act as proteasome substrates decreased sharply with increasing dendrimeric size. While the second generation of **4** (containing four terminal lysine residues) still showed moderate activity as a proteasome substrate, the third generation of **4** (containing eight terminal lysines) had no detectable activity (Figure 3b).

Generations 1−3 of compound **5**, which contained the full SQNY/PIVF linker, were then synthesized. These compounds showed size dependence similar to that of the various generations of **4**, with the third generation again showing no activity (Figure 3c). We then tested the third generation of **5** after incubation with HIV-1 protease. While the protease-free control had no activity and was identical to the DMSO control, the third generation of **5** showed significant activity as a proteasome substrate after protease cleavage. This demonstrates the ability of HIV-1 protease to rescue the activity of our compound (Figure 3d).

We have shown the first example of a cytotoxic small-molecule released from a "Trojan horse" compound by HIV-1 protease. We demonstrated the validity of our strategy using biotin and avidin as a steric cap, which prevents access of the cytotoxic inhibitor to the proteasome active site until activation by HIV-1 protease. Next, we demonstrated the feasibility of this strategy without exogenous proteins through the use of polylysine dendrimers. We showed that entry into the proteasome could be effectively blocked through the use of a third-generation lysine dendrimer, while activity remained in the first and second generations, in agreement with previous work showing that the proteasome can incorporate at least two peptide chains.[18] We were able to rescue the activity of our proteasome substrates through the addition of HIV-1 protease to our compounds containing a protease-labile linker. This work provides the foundation for the design of future conditional proteasome inhibitors for use in chemical biology studies of the proteasome and in the treatment of HIV and other diseases.

■ ASSOCIATED CONTENT

Supporting Information. Chemical and biochemical methods and characterization of novel compounds. This information is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
craig.crews@yale.edu

Present Addresses
[||]Department of Ophthalmology, Indiana University School of Medicine.

■ ACKNOWLEDGMENT

This work is funded by a grant from the Bill & Melinda Gates Foundation through the Grand Challenges Exploration Initiative. T.W.C. was the Canadian Institutes of Health Research Jean-François St-Denis Fellow in Cancer Research and a Bisby Fellow. N.A. was the American-Australian Association's Alcoa Foundation Fellow. The reagent EVA630, HIV protease, was obtained from the Programme EVA Centre for AIDS Reagents, NIBSC, UK, supported by the EC FP6/7 Europrise Network of Excellence, AVIP and NGIN consortia, and the Bill and Melinda Gates GHRC-CAVD Project and was donated by Dr. Iva Pitchova from the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Science, Praha, Czech Republic.

■ REFERENCES

(1) Mehellou, Y.; De Clercq, E. *J. Med. Chem.* **2010,** *53*, 521.
(2) Pomerantz, R. J.; Horn, D. L. *Nat. Med.* **2003,** *9*, 867.
(3) Geeraert, L.; Kraus, G.; Pomerantz, R. *Annu. Rev. Med.* **2008,** *59*, 487.
(4) Vocero-Akbani, A. M.; Heyden, N. V.; Lissy, N. A.; Ratner, L.; Dowdy, S. F. *Nat. Med.* **1999,** *5*, 29.
(5) Falnes, P. O.; Welker, R.; Kräusslich, H. G.; Olsnes, S. *Biochem. J.* **1999,** *343* (Pt. 1), 199.
(6) Turcotte, R. F.; Raines, R. T. *AIDS Res. Hum. Retroviruses* **2008,** *24*, 1357.
(7) Meng, L.; Mohan, R.; Kwok, B. H.; Elofsson, M.; Sin, N.; Crews, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **1999,** *96*, 10403.
(8) Groll, M.; Kim, K.-B.; Kairies, N.; Huber, R.; Crews, C. M. *J. Am. Chem. Soc.* **2000,** *122*, 1237.
(9) Löwe, J.; Stock, D.; Jap, B.; Zwickl, P.; Baumeister, W.; Huber, R. *Science* **1995,** *268*, 533.
(10) Moore, M. L.; Bryan, W. M.; Fakhoury, S. A.; Magaard, V. W.; Huffman, W. F.; Dayton, B. D.; Meek, T. D.; Hyland, L.; Dreyer, G. B.; Metcalf, B. W. *Biochem. Biophys. Res. Commun.* **1989,** *159*, 420.
(11) Elofsson, M.; Splittgerber, U.; Myung, J.; Mohan, R.; Crews, C. M. *Chem. Biol.* **1999,** *6*, 811.
(12) Zimmerman, M.; Ashe, B.; Yurewicz, E. C.; Patel, G. *Anal. Biochem.* **1977,** *78*, 47.
(13) Laidig, G. J.; Radel, P. A.; Smyth, M. S. U.S. Patent 2005/0256324 A1, November 17, 2005.
(14) Kaneshiro, T. L.; Wang, X.; Lu, Z.-R. *Mol. Pharmaceutics* **2007,** *4*, 759.
(15) Ohsaki, M.; Okuda, T.; Wada, A.; Hirayama, T.; Niidome, T.; Aoyagi, H. *Bioconjugate Chem.* **2002,** *13*, 510.
(16) Okuda, T.; Sugiyama, A.; Niidome, T.; Aoyagi, H. *Biomaterials* **2004,** *25*, 537.
(17) Watanabe, K.; Harada-Shiba, M.; Suzuki, A.; Gokuden, R.; Kurihara, R.; Sugao, Y.; Mori, T.; Katayama, Y.; Niidome, T. *Mol. BioSyst.* **2009,** *5*, 1306.
(18) Liu, C.-W.; Corboy, M. J.; DeMartino, G. N.; Thomas, P. J. *Science* **2003,** *299*, 408.